Evidence of Cardiocyte Apoptosis in Myocardium of Dogs with Chronic Heart Failure

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It is often speculated that progressive deterioration of left ventricular function in heart failure is due to ongoing loss of viable cardiocytes. In this study, we examined the possibility that cardiocyte loss in heart failure may be due, in part, to apoptosis, an active process of gene-directed cellular self-destruction. Studies were performed in left ventricular tissue obtained from 10 dogs with chronic heart failure produced by multiple intracoronary microembolizations (left ventricular ejection fraction 27 ± 1%) and from 5 normal dogs. Evidence for cardiocyte apoptosis was based on transmission electron microscopy criteria and on in situ immunohistochemical labeling of nuclear DNA fragmentation. There was no evidence of apoptotic cardiocytes in normal dogs. Features of cardiocyte apoptosis were observed in dogs with heart failure primarily in regions bordering old infarcts. Electron microscopic features of cardiocyte apoptosis included (1) intact sarcolemma and inner organelles in the presence of compaction and segregation of nuclear chromatin into sharply delineated masses that abut the nuclear envelope, (2) intact sarcolemma in the presence of cytoplasm shrinkage, blebbing, and nuclear fragmentation, and (3) intact sarcolemma in the presence of complete disorganization of inner organelles and disappearance of nucleolemma. A count of all of the apoptotic bodies positively labeled for nuclear DNA fragments showed that 11% were of cardiocyte origin confirmed by positive labeling with striated muscle antimyosin antibody. We conclude that morphological and biochemical features of cardiocyte apoptosis exist in the left ventricular myocardium of dogs with chronic heart failure.   (Am J Pathol 1996, 148:141–149)

Progressive deterioration of left ventricular function is a characteristic feature of the heart failure state. This progressive deterioration often occurs despite the absence of intercurrent adverse clinical events such as ischemia or infarction. The exact mechanisms that underlie this process are not known. A possible working hypothesis is that progressive left ventricular dysfunction occurs, in part, as a result of ongoing loss of viable cardiocytes. There is no direct evidence to date, however, supporting the concept that ongoing death of cardiocytes occurs in chronic heart failure. Electron microscopic studies on tissues obtained from hypertrophied and failing human hearts[1-6] and hearts of dogs with experimentally induced chronic heart failure[7,8] have clearly established the existence of myocyte degeneration. These observations provide some support, albeit indirect, to the concept of ongoing myocyte loss in the failing heart.

Two mechanisms of cell death have been described to date, namely, death through necrosis and death through apoptosis. The distinguishing features of cell necrosis are membrane rupture with associated inflammation.[9-11] Characteristic ultrastructural features of cardiocyte necrosis include cell swelling, plasma membrane breakdown, clumping of nuclear chromatin into ill-defined masses, gross swelling and disruption of sarcoplasmic reticulum and mitochondria, and the appearance of flocculent or granular densities in the matrix of mitochondria.[12,13] As a consequence of sarcolemmal rupture, calcium overload and lethal disturbances in other electrolytes occur in necrotic cardiocytes.[14,15] In contrast, apoptosis occurs in the absence of membrane rupture and inflammation and is characterized by fragmentation of nuclear DNA.[16-18] Early ultrastructural manifestation of apoptosis include compaction and segregation of nuclear chromatin into sharply delineated

masses that lie against the nuclear envelope, condensation of the cytoplasm, and mild convolution of the nuclear and cellular outlines. Subsequently, nuclear fragmentation occurs and the cell surface develops pediculated protuberances and separate into membrane-bound apoptotic bodies, which are phagocytosed or digested by adjacent cells. The apoptotic cell does not rupture before it is engulfed by a macrophage.[17,18] In contrast to passive necrosis, which occurs in response to lethal injury, apoptosis appears to be an active, strongly regulated, energy-requiring process that appears to be under genetic control. That is why the terms apoptosis and programmed cell death are often interchangeable.[19-21] In normal adult tissues, apoptosis plays a crucial role in the maintenance of proliferating cell populations and is balanced by mitosis.[22,23] It is now recognized that terminally differentiated cells may also retain the ability to die by apoptotic mechanisms.[24] Elements of cardiocyte apoptosis have been demonstrated in patients treated with certain anticancer drugs,[25] in ischemia/reperfusion studies of rabbit myocardium,[26] and in cultured neonatal rat cardiocytes exposed to hypoxic conditions.[27] In the present study, we examined whether morphological and biochemical features of cardiocyte apoptosis exist in myocardium of dogs with chronic heart failure.

Materials and Methods

Experimental Preparation

Left ventricular myocardial samples from 15 dogs were used in the study. Dogs weighed between 20 and 30 kg; 10 dogs had chronic heart failure and 5 were normal. Chronic heart failure was produced by multiple sequential intracoronary embolization with polystyrene latex microspheres (77 to 102 μm in diameter) as previously described.[28] Coronary microembolizations were performed during sequential cardiac catheterizations under general anesthesia and sterile conditions. The anesthesia regimen consisted of a combination of intravenous oxymorphone hydrochloride (0.22 mg/kg), diazepam (0.17 mg/kg), and sodium pentobarbital (150 to 250 mg to effect). Embolizations were discontinued when left ventricular ejection fraction, measured angiographically, was 30 to 40%. Two weeks after the last embolization, left ventricular ejection fraction was 33 ± 1%. Subsequently, dogs were maintained for a period of 3 to 4 months without any additional interventions or therapy of any kind. At the end of this follow-up period, dogs underwent a cardiac catheterization with left

ventriculography to assess ejection fraction.[29] The ejection fraction before sacrifice was 27 ± 1%, which was significantly lower than two weeks after the last embolization (P < 0.001). At the end of this procedure, while still under general anesthesia, the chest was opened through a left thoracotomy, the pericardium was opened, and the heart was rapidly removed and immediately placed in ice-cold cardioplegic solution. The study was approved by the institution's Care of Experimental Animals Committee and conformed to the guiding principles of the American Physiological Society.

Transmission Electron Microscopy

Transmural tissue samples, approximately 1 mm thick, were obtained from the left ventricular free wall at the mid-ventricular level and immediately immersed in ice-cold 3.5%, 0.1 mol/L phosphate-buffered glutaraldehyde (pH 7.2). After 24 hours, the tissue blocks were postfixed for 2 hours in 1%, 0.1 mol/L phosphate-buffered OsO_4 (pH 7.2), and subsequently embedded in araldite. Areas of interest were selected from toluidine-blue-stained semithin sections. Ultrathin sections were mounted in slot grids, covered with Formvar, double stained in uranyl acetate and lead citrate. Sections were examined in a Philips 201 transmission electron microscope at magnifications ranging from ×3,000 to ×15,000 to assess ultrastructural features of cardiomyocytes.

In Situ *Identification of Nuclear DNA Fragmentation*

Transmural samples from the left ventricular free wall were mounted on cork with Tissue-Tek embedding media, frozen in isopentane cooled to −160°C in liquid nitrogen and stored at −70°C until ready for use. Cryostat sections were stained with the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD) to detect cells showing nuclear DNA fragmentation. The procedure is based upon the method described by Schmitz et al.[30] Residues of digoxigenin-nucleotide are catalytically added to the DNA by terminal deoxynucleotidyl transferase, an enzyme that catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA. The anti-digoxigenin antibody fragment carries either a fluorescein or peroxidase to the reaction site. For negative control to DNA fragmentation labeling, the serial sections were stained without terminal de-

oxynucleotidyl transferase. To identify cells or bodies of cardiocyte origin, sections stained for DNA fragments with the fluorescein label were then double stained overnight at 4°C with a monoclonal mouse striated muscle antimyosin antibody (working dilution 1:10,000). A sheep anti-mouse biotinylated immunoglobulin G (Sigma Chemical Co., St. Louis, MO; working dilution 1:600) was used as the secondary antibody. For visualization, sections were stained with streptavidin-Texas Red (Sigma; working dilution 1:400). From each section, 20 light microscopic fields (×40) were used to count the number of cells positively labeled for nuclear DNA fragmentation. Ten fields were selected at random from myocardial regions bordering old infarctions and 10 fields from myocardial regions remote from any infarction. The average number of myocytes in fields selected from regions bordering old infarcts was 89 (range 66 to 120) and 113 (range 88 to 140) in fields selected from remote myocardial regions. Transmural section from the left ventricular free wall from five normal dogs were prepared and examined in an identical fashion. In these normal dogs, only 10 fields were selected at random for quantitation of the number of bodies manifesting DNA fragmentation. The average number of cardiocytes in these fields was 149 (range 144 to 158). Only sections stained for fluorescein-labeled DNA fragmentation events were used for quantitative analysis. The sections stained for DNA fragmentation labeled with peroxidase were double stained with methyl green and were used for qualitative analysis. For negative control to myosin staining, serial sections were stained without antimyosin antibody.

Data Analysis

Transmission electron microscopy was evaluated qualitatively for the presence of specific features of apoptosis. The number of bodies manifesting nuclear DNA fragmentation per field were compared between normal and failed left ventricular tissue using a t-test for two means. For this test, a probability of 0.05 or less was considered significant. In heart failure dogs, the number of bodies manifesting DNA fragmentation per field were compared between left ventricular regions bordering old infarcts and left ventricular regions remote from any infarcts by Student's paired t-test. For this test, a probability of 0.05 or less was considered significant. All values are reported as the mean ± SEM.
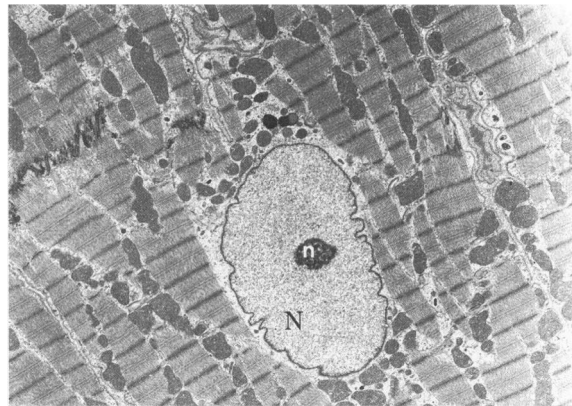

Figure 1. *Electron micrograph of a cardiocyte from the left ventricle of a normal dog. The nucleus (N) shows even distribution of chromatin and a normal nucleolus (n). Uranyl acetate and lead citrate; final magnification, × 5400.*

Results

Transmission Electron Microscopy

Electron microscopic features of cardiocyte apoptosis were identified in left ventricular tissue obtained from every dog with heart failure but in none of the tissue specimens obtained from the left ventricles of normal dogs. In heart failure dogs, the majority of cardiocytes with features consistent with apoptosis were located in left ventricular regions bordering old infarcts. In most instances, cardiocytes showing features of apoptosis were encircled by large amounts of collagen and were always present in the absence of any inflammatory response. Figure 1 illustrates a typical cardiocyte from the left ventricle of a normal dog. The nucleus shows even distribution of chromatin and a normal nucleolus. A typical cardiocyte from the left ventricle of a heart failure dog remote from any old infarcts also shows evenly distributed nuclear chromatin and a normal nucleolus (Figure 2).

In heart failure dogs, the majority of cardiocytes that were located in zones bordering old infarcts showed modest margination and clumping of nuclear chromatin into ill-defined masses that are present throughout the nuclear matrix. This feature is consistent with the possible presence of mild ischemia or hypoxia as previously described (Figure 3).[12–14] Compaction of nuclear chromatin into sharply circumscribed, uniformly dense masses that abut the nuclear envelope was seen in occasional cardiocytes (Figures 4 and 5). In these cells, the inner organelles were preserved and the sarcolemma appeared intact. According to the ultrastructural features of apoptosis described earlier,[17–18] these cells most likely represent an early stage of apoptosis. Figure 6 is a typical example of a cardio-
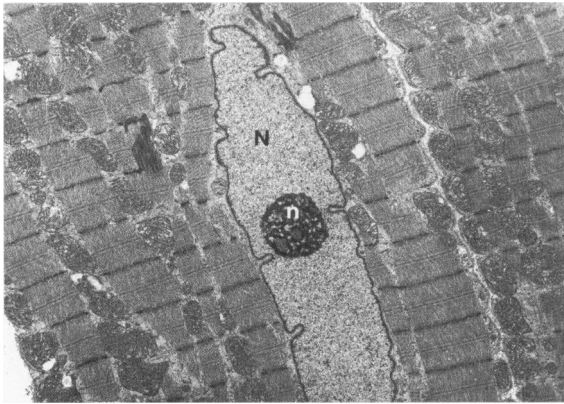
Figure 2. *Electron micrograph of cardiocyte from left ventricle of dog with heart failure. The cell is located remote from old infarct. The nucleus (N) shows even distribution of chromatin and a normal nucleolus (n). Uranyl acetate and lead citrate; final magnification, ×9000.*

Figure 4. *Electron micrograph of cardiocyte from the left ventricle of a dog with chronic heart failure manifesting the earliest stage of apoptosis. The cardiocyte is located on the border of an old infarct and encircled by collagen (CL). The nucleus (N) shows normal shape and contains small sharply delineated electron dense chromatin masses (**arrowhead**) that abut the nuclear envelope. Clumped ill defined nuclear chromatin is also present (**arrow**). Mitochondria (M) and myofibrils (MF) appear normal. The sarcolemma (SM) appears intact. Uranyl acetate and lead citrate; final magnification, ×12,400.*

cyte in an advanced stage of apoptosis. This cell showed shrinkage of the cytoplasm and a vast amount of sarcolemmal protrusions and invaginations. The nucleus is fragmented and electron dense and exhibits compaction of nuclear chromatin into sharply delineated masses that lie against the nuclear envelope, whereas the sarcolemma itself appears to be intact (Figure 6). Figure 7 illustrates the end stage of the apoptotic process, namely, the engulfment of an intact membrane-bound apoptotic body by a macrophage. In this body, the nucleolemma is absent and the apoptotic body shows just electron-dense, sharply delineated fragments of nuclear chromatin. At this stage, it is not possible to judge the origin of the apoptotic body only on the basis of its electron microscopic appearance.
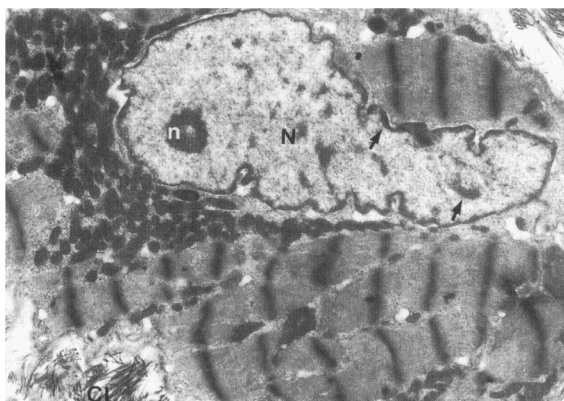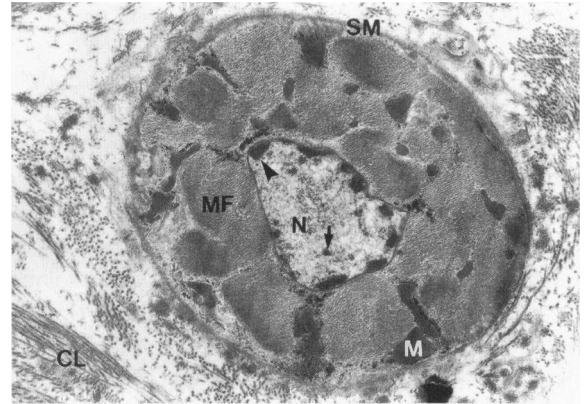
In Situ *Nuclear DNA Fragmentation*

Figure 8a shows a positive peroxidase staining for nuclear DNA fragmentation of a cardiocyte encircled by collagen in a region bordering an old infarct. Negative control to DNA fragmentation staining (staining for DNA fragmentation without terminal deoxynucleotidyl transferase) confirmed the high specificity of the method selected for DNA fragmentation labeling. Similarly, negative control staining without antimyosin antibody also did not show any nonspecific binding of the secondary antibody. Typical examples of cardiocytes and small bodies of cardio-
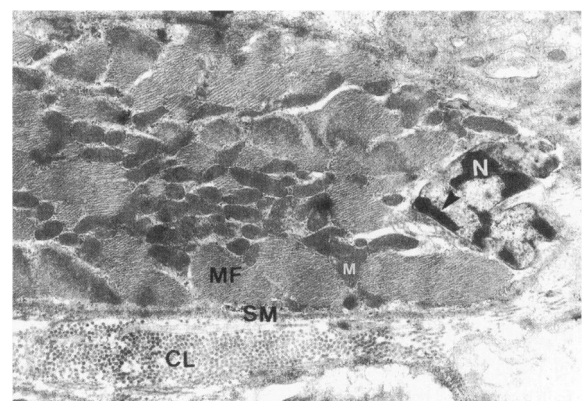

Figure 3. *Electron micrograph of cardiocyte from left ventricle of dog with heart failure. The cell is located in a region bordering an old infarct. The cardiocyte is encircled by collagen (CL) and shows mild margination and clumping of ill defined nuclear chromatin (**arrow**) consistent with moderate ischemia or hypoxia. N, nucleus; n, nucleolus. Uranyl acetate and lead citrate; final magnification, ×12,400.*

Figure 5. *Electron micrograph of cardiocyte from the left ventricle of a dog with chronic heart failure manifesting a more advanced stage of apoptosis compared with the cardiocyte in Figure 4. The cardiocyte is located on the border of an old infarct and is encircled by collagen (CL). The nucleus (N) shows deep invaginations of the nucleolemma and contains large sharply delineated electron dense chromatin masses (**arrowhead**) that abut the nuclear envelope. Mitochondria (M) and myofibrils (MF) appear normal. The sarcolemma (SM) appears intact. Uranyl acetate and lead citrate; final magnification, ×17,500.*
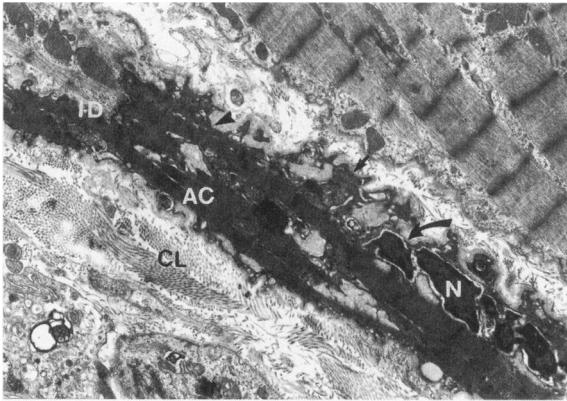
Figure 6. *Electron micrograph of cardiocyte from the left ventricle of a dog with chronic heart failure manifesting a more advanced stage of apoptosis compared with the cardiocyte in Figure 5. The cardiocyte is located on the border of an old infarct and is encircled by collagen (CL). The cytoplasm is electron dense and shows shrinkage. The sarcolemma manifests deep invaginations (arrowhead) and protrusions (arrow) indicative of considerable blebbing. The electron-dense nucleus (N) is fragmented and shows sharply delineated electron-dense chromatin that abuts the nuclear envelope (curved arrow). The sarcolemma appears intact. ID, intercalated disc; AC, apoptotic cell. Uranyl acetate and lead citrate; final magnification, ×12,400.*

cyte origin with positive fluorescein labeling for nuclear DNA fragmentation are shown in Figure 8, a–f. The majority of apoptotic events observed in this study, based on labeling for nuclear DNA fragmentation, were small bodies of cardiocyte origin. Identification of cardiocytes with positive labeling of the nucleus for DNA fragmentation was a much rarer event.

In left ventricular tissue from normal dogs, the number of apoptotic bodies per field regardless of cell origin was not significantly different than the number of apoptotic bodies located in remote myo-
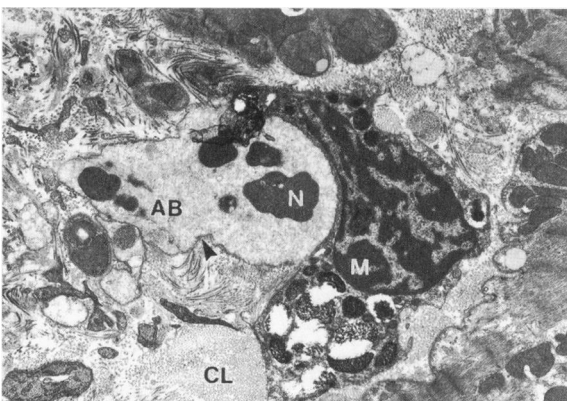
Figure 7. *Electron micrograph of an apoptotic body (AB) being engulfed by a macrophage (M). The micrograph was obtained from the left ventricle of a dog with chronic heart failure. The apoptotic body was located on the border of an old infarct. The body appears to have an intact membrane (arrowhead) and contains fragments of sharply delineated electron-dense nuclear chromatin without a nuclear envelope (N) and without any other recognizable cytoplasm organelles. CL, collagen. Uranyl acetate and lead citrate; final magnification, ×12,400.*

cardium of failing hearts (Table 1). In normal left ventricular tissue, none of the apoptotic bodies could be ascribed to cardiocytes as none could be confirmed to contain myosin based on labeling with antimyosin antibody. In contrast, in failed left ventricular tissue remote from any old infarcts, approximately 2.7% of all apoptotic bodies were of cardiocyte origin based upon positive labeling with antimyosin antibody (Table 1). In heart failure dogs, the majority of apoptotic bodies identified by labeling for nuclear DNA fragmentation were present in left ventricular regions bordering old infarcts (Table 1). In regions bordering old infarcts, the number of apoptotic bodies per field, regardless of cell origin, was significantly greater than the number of apoptotic bodies per field in left ventricular regions remote from any infarcts (Table 1).

Discussion

The results of the present study demonstrate that morphological and biochemical features of apoptosis exist in cardiocytes of dogs with chronic heart failure. These observations provide, for the first time, some evidence for cardiocyte apoptosis in heart failure. The confirmation of this process was based on established electron microscopic criteria for apoptosis and on the presence of nuclear DNA fragmentation, a commonly accepted key marker of the apoptosis process.

To date, two types of cell death have been identified. The most familiar is necrosis, which generally follows an acute injury such as ischemia. The other is termed apoptosis or programmed cell death and was first described by Kerr et al.[17] Necrosis is characterized by cell swelling followed by membrane rupture that, in turn, evokes an inflammatory response.[12,31] In contrast to necrosis, apoptosis is an active, tightly regulated, energy-requiring process that appears to be under genetic control.[17,32] Apoptotic cells do not swell but actually shrink.[18] These cells also exhibit nuclear fragmentation and chromatin condensation with lysis of the nucleolemma whereas the sarcolemma remains intact until the cell is engulfed by local macrophages with avoidance of inflammation.[31] Apoptotic cardiocytes identified in the present study showed similar features, namely, marked cytoplasm shrinkage and absence of inflammation while the sarcolemma appeared intact. In the present study we also demonstrated at the electron microscopic level the rarely seen event of an apoptotic body of unknown origin being engulfed by a macrophage.
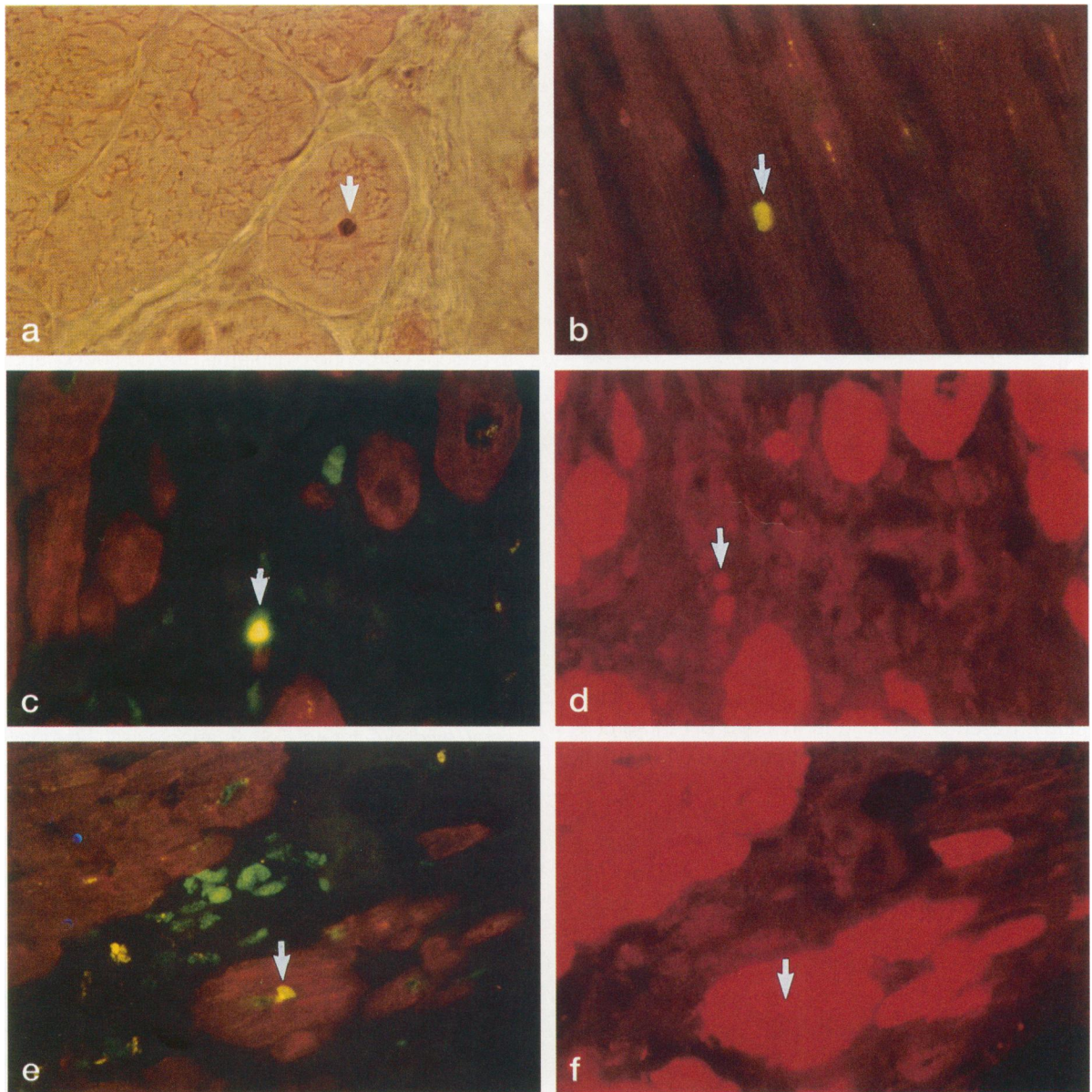
Figure 8. *Sections of left ventricular myocardium of a dog with chronic heart failure. Final magnification, × 500.* **a:** *Region bordering an old infarct. Nuclear DNA fragmentation staining with peroxidase. Counterstaining with methyl green.* **Arrow** *indicates the cardiomyocyte showing positive reaction for nuclear DNA fragmentation.* **b:** *Region remote from an old infarct. Section stained for nuclear DNA fragmentation with a fluorescein label.* **Arrow** *indicates the cell with the positive labeling for nuclear DNA fragmentation.* **c:** *Region bordering an old infarct. Section stained for nuclear DNA fragmentation with the fluorescein label.* **Arrow** *indicates positive staining for nuclear DNA fragmentation.* **d:** *Same section as in* **c** *stained with antimyosin antibody. One of the apoptotic bodies, seen in* **c** *shows positive staining for myosin* (**arrow**) *confirming its cardiocyte origin.* **e:** *Region bordering an old infarct. Section stained for nuclear DNA fragmentation with the fluorescein label.* **Arrow** *indicates positive staining for nuclear DNA fragmentation.* **f:** *Same section as in* **e** *stained with antimyosin antibody. One of the cells, seen in* **e** *shows positive staining for myosin* (**arrow**) *confirming its cardiocyte origin. Small yellow dots that are visible in* **b** *and* **e** *are most likely the result of nonspecific staining of lipofuscin bodies.*

In 1987, Wyllie[33] proposed four cardinal elements involved in apoptosis. These included (1) cell volume reduction, (2) nuclear chromatin condensation in early stages of apoptosis, (3) changes in the cell membrane that allow recognition by phagocytic cells, and (4) dependence of the apoptotic process on active protein synthesis.[33] In the present study, most of these features were identified in cardiocytes.

We observed a clear reduction in the size of apoptotic cardiocytes, condensation of nuclear chromatin into sharply delineated masses that abut the nuclear envelope, and changes in the sarcoplasm characterized by shrinkage and blebbing. All of these features, however, were not always identified in each affected cardiocyte. The formation of blebs is thought to be one of the steps of the apoptotic pro-

Table 1. *Number of Cells per Field (×40) Showing Nuclear DNA Fragmentation in Left Ventricular Myocardium of Normal Dogs and Dogs with Heart Failure*

	Normal dogs	Heart failure dogs	
		Remote region	Infarct border
All cells regardless of origin	0.94 ± 0.16	0.75 ± 0.08	3.21 ± 0.42*
Cells of cardiocyte origin	0	0.02 ± 0.01	0.41 ± 0.05*

*$P < 0.001$; remote region *versus* infarct border.

cess.[34] The blebs fragment into characteristic apoptotic bodies that, nonetheless, retain structural integrity.[34] Such membrane-bound apoptotic bodies are short lived and are phagocytosed.[35] Accordingly, each apoptotic body identified in the present study as being of cardiocyte origin because of the presence of myosin in the cytoplasm represents an isolated part of a former cardiocyte. It is likely that many apoptotic bodies originate from a single cardiocyte through the blebbing process. On the other hand, it is not known how many apoptotic bodies that do not contain myosin are of myocyte or non-myocyte origin. For this reason, and because the duration of the apoptotic process is not known for cardiocytes, one cannot determine, at present, the rate of the cardiocyte apoptosis process in heart failure. We can, however, state with some confidence that, in this dog model of heart failure, the apoptotic process appears to be substantially more active in left ventricular regions bordering old infarcts in comparison to regions remote from any infarcts.

Most of our present knowledge of the nature of apoptosis has come from studies with cells in culture in which apoptosis was experimentally induced.[3] These experiments showed that apoptosis is associated with increased endogenous endonuclease activity[36] with DNA cleavage being the most characteristic biochemical feature of this process.[37] Accordingly, the appearance of the ladder of nucleosomal DNA fragments in agarose gels became the hallmark of apoptosis or programmed cell death. Because apoptosis tends to involve single cells, standard detection methods involving genomic DNA extraction are not useful as the analysis does not allow distinction between individual cells.[21] For this reason, we selected an immunohistochemical staining procedure for *in situ* visualization of nuclear DNA fragmentation.

Studies by other investigators have suggested that cardiocytes may undergo apoptosis under certain pathophysiological conditions. Many of these studies, although important, provide only limited evidence of cardiocyte apoptosis. Indirect evidence of apoptosis, based on phagocytosis of a cardiocyte was described by Bing[32] in a spontaneously hypertensive rat with left ventricular hypertrophy. James[31] presented transmission electron microscopic evidence of apoptosis in cells of the sinus node obtained from hearts of two patients treated surgically for long QT syndrome. In early stages of myocardial ischemia in the rat, Barr and Tomei[24] presented a transmission electron microscopic example of an apoptotic cardiocyte with a typical feature of sarcolemmal blebbing. In a rabbit model of myocardial ischemia and reperfusion, Gottlieb et al[26] presented evidence of cardiocyte apoptosis during reperfusion of ischemic myocardium. Evidence was based on electron microscopic findings of nuclear chromatin condensation and DNA ladder.[26]

The factors that may trigger apoptosis in the failed myocardium are not fully understood. The possibility exists that apoptosis may be induced by the same agents that produce necrosis with the type of cell death being dependent on the severity of the insult rather than its qualitative nature.[35] Some evidence exists that suggests that increased cytosolic calcium concentration,[38] formation of oxygen free radicals, temporary ischemia,[26] and cell exposure to hypoxia[27] may each be a trigger for apoptosis. One cannot exclude the possibility that some of these triggers and perhaps all may exist in the failing heart. In myocytes bordering old infarcts, we showed electron microscopic evidence of the possible existence of mild ischemia or hypoxia of collagen-encircled cardiocytes based on the observation of ill-defined nuclear chromatin aggregation (Figure 3). This finding is not likely to be a consequence of tissue fixation by immersion as all of the cells of normal dogs and all of the cardiomyocytes from failing dogs that are not surrounded by an excessive amount of collagen do not show any signs of ischemic injury. In the left ventricle of dogs with chronic heart failure, we previously demonstrated that myocytes with advanced degeneration were encircled by extensive amounts of collagen and were located in myocardial regions bordering old infarcts.[39] These cardiocytes also manifested a near twofold increase in lactate dehydrogenase activity compared with left ventricular regions that manifested little or no interstitial fibrosis.[40] These findings suggest that chronic hypoxia or ischemia, a potential trigger of apoptosis, may be present in the failing left ventricle at least in regions where the cells are encircled by large amounts of collagen. In the present study, apoptotic cardiocytes were almost invariably encircled by extensive colla-

gen deposits and occurred with the highest frequency in myocardial regions bordering old infarcts.

Significance and Conclusions

The results of the present study indicate that morphological and biochemical features of apoptosis exist in cardiocytes in dogs with chronic heart failure. The highest incidence of apoptotic events occurred in myocardial regions bordering old infarctions. The importance of these findings rests on the demonstration that cardiocytes, which are believed to be terminally differentiated, may undergo apoptosis. The possibility that cardiocyte apoptosis can occur in the setting of chronic heart failure provides a potential mechanism for ongoing loss of functional cardiac units in this disease state.

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